In vivo evidence that the lipid-regulating activity of the ACAT inhibitor CI-976 in rats is due to inhibition of both intestinal and liver ACAT

B. R. Krause,[1] M. Anderson, C. L. Bisgaier, T. Bocan, R. Bousley, P. DeHart,* A. Essenburg, K. Hamelehle, R. Homan, K. Kieft, W. McNally,* R. Stanfield, and R. S. Newton

Departments of Pharmacology and Pharmacokinetics/Drug Metabolism,* Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105

Abstract CI-976, a new trimethoxy fatty acid anilide, is a potent and specific inhibitor of liver and intestinal acyl coenzyme A:cholesterol acyltransferase (ACAT) in vitro. Several in vivo approaches were used to determine the efficacy and sites of action of this compound in rats. CI-976 decreased non-high density lipoprotein (HDL)-cholesterol and increased HDL-cholesterol in rats with pre-established dyslipidemia. High performance gel chromatographic separation of plasma lipoproteins also revealed that CI-976, but not CL 277,082, lowered low density lipoprotein (LDL)-cholesterol and elevated HDL-cholesterol. Bay o 2752, octimibate, melinamide, and SaH 58-035 were all less potent in vivo compared to CI-976 and CL 277,082, and CI-976 produced the greatest decrease in liver cholesteryl esters. Subcutaneous (SC) administration of CI-976 was also efficacious in cholesterol-fed animals. In sucrose-fed rats, oral and SC CI-976 administration potently lowered plasma triglycerides. Hepatic cholesteryl ester accumulation in the ethinyl estradiol-treated rat was also diminished by orally administered CI-976. ACAT activity and cholesteryl ester mass were dose-dependently decreased in the livers from cholesterol-fed rats treated with CI-976, suggesting a direct effect on the liver. In both hypercholesterolemic and hypertriglyceridemic models, CI-976 also decreased plasma apoB concentrations. In other experiments radiolabeled CI-976 accumulated in the liver after multiple doses. Time-dependent changes in biliary lipid and bile acid secretion suggested that free cholesterol did not accumulate in the liver but instead was excreted as such or as bile acid. Finally, inhibition of endogenous and exogenous intestinal cholesterol absorption was demonstrated using several in vivo techniques. The combined data strongly supports the hypothesis that orally administered CI-976 inhibits both intestinal and hepatic ACAT, and that both of these enzymes may be determinants of plasma lipid concentrations in the rat.—**Krause, B. R., M. Anderson, C. L. Bisgaier, T. Bocan, R. Bousley, P. DeHart, A. Essenburg, K. Hamelehle, R. Homan, K. Kieft, W. McNally, R. Stanfield, and R. S. Newton.** In vivo evidence that the lipid-regulating activity of the ACAT inhibitor CI-976 in rats is due to inhibition of both intestinal and liver ACAT. *J. Lipid Res.* 1993. **34:** 279–294.

Supplementary key words cholesterol • lipoproteins • atherosclerosis

It has been repeatedly demonstrated that various specific inhibitors of intestinal ACAT (acyl-CoA:cholesterol acyltransferase, EC 2.3.1.1.26, **Fig. 1**) can inhibit the intestinal absorption of cholesterol in experimental animals. Melinamide (1), SaH 58-035 (2), Bay o 2752 (3), CL 277,082 (4), and octimibate (5) all decrease cholesterol output into rat mesenteric lymph without altering triglyceride absorption. Inhibition of cholesterol absorption by ACAT inhibitors has also been demonstrated in rabbits using the dual-isotope method (6), and in rats (7) and hamsters (8) by isotope-fecal methods. However, data on the relative efficacy of these compounds with respect to changes in plasma cholesterol or cholesterol distribution upon oral dosing are not available. In fact, dose-response data for more than one compound have not been obtained in any single experiment in rats in which acute or chronic efficacy (i.e., plasma cholesterol-lowering activity) was examined. As virtually all studies assume that inhibition of cholesterol absorption is the sole mechanism for ACAT inhibitors, no effort has been made to determine whether liver ACAT may also account, at least in part, for the observed efficacy of ACAT inhibitors in the rat.

In the present report, we further extend previously published information (9, 10) on the in vitro activity of a new trimethoxy anilide ACAT inhibitor, CI-976 (Fig. 1). In addition, we provide a more detailed evaluation of its in vivo activity in several rat models. The new informa-

Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; apo, apolipoprotein; CEH, cholesterol esterase; DTNB, dithiobis-2-nitrobenzoic acid; HPLC, high performance liquid chromatography; LCAT, lecithin:cholesterol acyltransferase; MGAT, acyl-coA:monoglyceride acyltransferase; SC, subcutaneous; IV, intravenous.

[1]To whom correspondence should be addressed.

tion derived from the study of this compound supports the notion that inhibition of both intestinal and liver ACAT contribute to the observed efficacy for absorbable ACAT inhibitors in this animal species.

METHODS AND MATERIALS

In vitro activity

In vitro ACAT inhibitory activity was determined in hepatic or intestinal microsomes prepared from rabbits fed chow supplemented with 2% cholesterol and 10% safflower oil for 6 weeks. Data are also presented using microsomes from the livers of rats fed chow supplemented with 5.5% peanut oil, 0.5% cholic acid, and 1.5% cholesterol for 2 weeks (PCC diet). The assay was a modification of that described by Field and Salome (11) and has recently been described in detail (10). Briefly, each assay contained 0.2 mg of microsomal protein and fatty acid-poor bovine serum albumin (3 mg/ml) in 0.04 M KH_2PO_4 buffer, pH 7.4, containing 0.05 M KCl, 0.03 M EDTA, and 0.3 M sucrose. Drug dilutions were made in DMSO (5 μl DMSO/200 μl total incubation volume). As reported by others (3), DMSO did not affect ACAT activity under these conditions. The reaction was started by the addition of [^{14}C]oleyl-CoA (50 μM, 7 dpm/pmol), and after 3 min the reaction was stopped by the addition of chloroform–methanol 2:1. [^{3}H]cholesteryl oleate was used

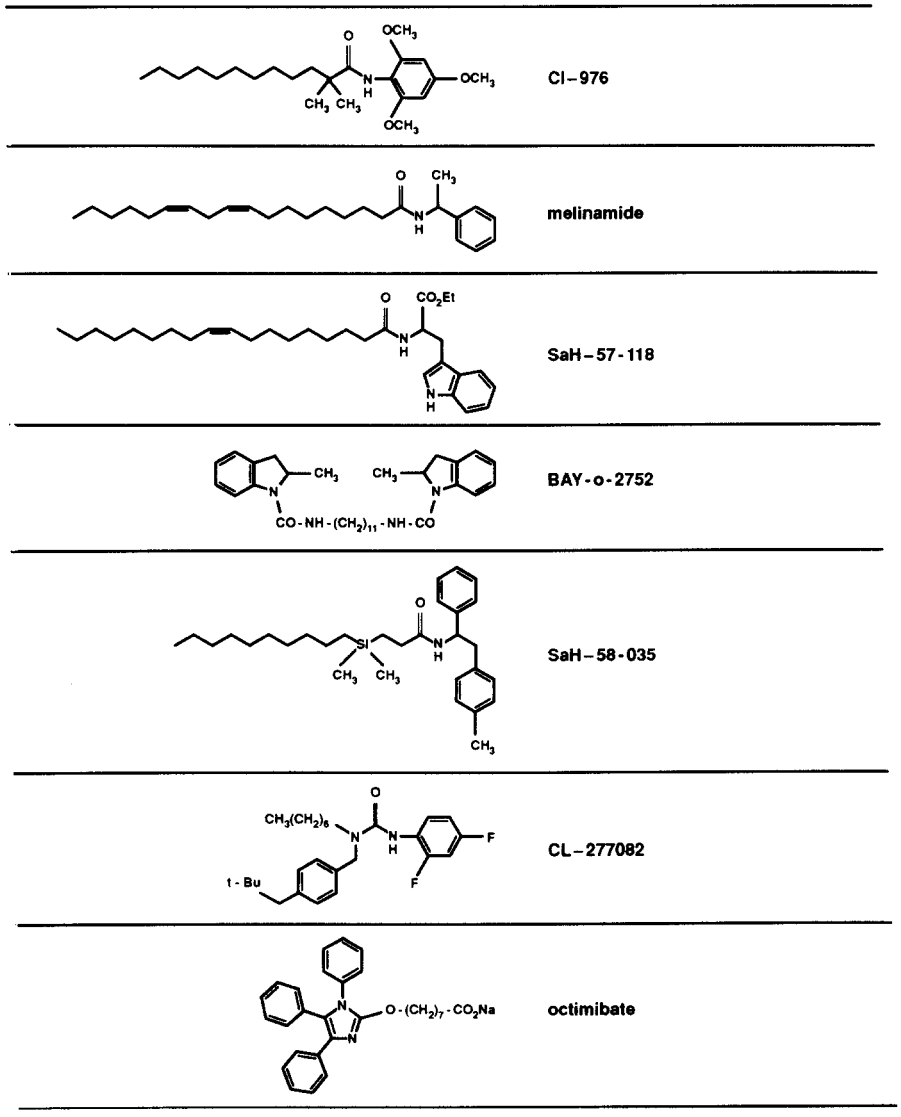

Fig. 1. Chemical structures of selected ACAT inhibitors.

as an internal standard. Lipid extracts were dissolved in chloroform, plated on TLC plates (silica gel G), and developed in hexane–petroleum ether–acetic acid 80:20:1. Unlabeled, carrier cholesteryl oleate was added to the internal standard to aid band visualization with I_2 vapor. The band corresponding to cholesteryl esters was then scraped into scintillation vials and radioactivity was determined by liquid scintillation spectroscopy. For each compound four concentrations were evaluated in duplicate. IC_{50} values were determined by performing a nonlinear least-squares fit of the data to a log dose-response curve.

Specificity of inhibition by CI-976

Field et al. (9) previously demonstrated that CI-976 was a specific inhibitor of ACAT in CaCo-2 cells. Specificity of CI-976 for ACAT inhibition was further addressed by evaluating its effects on a variety of rat esterifying enzymes. Acyl-CoA:monoglyceride acyltransferase (MGAT) activity was assayed in triplicate using rat intestinal microsomes (2, 12). Each assay tube contained drug in 5 μl of DMSO or DMSO alone, 120 μl Tris-HCl buffer (0.1 M, pH 8.0), 20 μl of 10% bovine serum albumin in Tris-HCl buffer, monoolein (10 μl in ethanol, 25 nmol), and microsomal protein (20 μl, 10 μg). After a 5-min, 30°C preincubation, the reactions were started by the addition of [^{14}C]oleoyl-CoA (50 nmol, 1500 dpm/nmol). All other steps were as described above for the ACAT assay, except that the diolein band, visualized with carrier, was identified and counted. Cholesterol esterase (CEH) synthetic activity was assayed according to the method described by Gallo (13). Briefly, cytosols were obtained from rat intestinal scrapings and incubated with [^{14}C]cholesterol, oleic acid, sodium taurocholate, and albumin in phosphate buffer (pH 6.2) for 3 h at 37°C. The reaction was stopped by adding chloroform–methanol 2:1, and [^{3}H]cholesteryl oleate and carrier cholesteryl oleate were added. The reaction product ([^{14}C]cholesteryl ester) was isolated and counted as above. Finally, the effect of CI-976 on lecithin:cholesterol acyltransferase (LCAT) was determined by incubating serum (rat) in phosphate buffer (0.2 M, pH 7.1) with an albumin-[^{14}C]cholesterol emulsion for 4 h at 37°C (14). *p*-Nitrophenyl-N-butyl carbamate and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), known inhibitors of CEH (15) and LCAT (14), were used as positive controls for the CEH and LCAT assays, respectively.

Efficacy in cholesterol-fed rats

Male Sprague-Dawley rats (200–225 g) were used for all experiments except where specifically indicated otherwise. Drugs were administered by daily oral gavage using aqueous carboxymethylcellulose/Tween-20 (1.5%/0.2%) suspensions (6). In some experiments compounds were administered daily by subcutaneous injection using an ethanol–polyethylene glycol 10:90 (v/v) vehicle. In all experiments control animals received vehicle alone by the appropriate route. Efficacy was assessed by the ability of the drugs to reduce diet-induced hypercholesterolemia after multiple doses. Rats were fed the PCC diet for 2 weeks with drug dosing during the second week only (i.e., treatment of preestablished hypercholesterolemia). In some experiments drug treatment and PCC diet were started on the same day and continued for 5–7 days (chronic prevention of dyslipidemia). This protocol was used to study differences between drug routes, sex differences, ex vivo ACAT activity, and biliary secretion of lipids and bile acids (see below). In all cholesterol-fed rat experiments animals were killed 18–24 h after the last dose in the nonfasted state. Plasma and liver cholesterol concentrations were determined by standard enzymatic procedures, as described previously (16). Cholesterol distribution among lipoproteins in selected experiments was determined either by the dextran-sulfate precipitation method (17) or by high-performance gel chromatography using Superose 6HR and the Beckman Gold HPLC System (18). The latter method fractionates microliter quantities of whole plasma into VLDL, LDL, and HDL-sized particles. The cholesterol content of these particles is determined by a postcolumn reaction with an enzyme-based cholesterol reagent. In one experiment cholesterol-fed rats were dosed orally with CI-976 for 5 days; then the livers were removed and ACAT activity was assessed in freshly prepared crude liver homogenates rather than microsomes essentially as described by Gallo, Wadsworth, and Vahouny (19) with minor modifications. [^{14}C]oleoyl-CoA was used as the radiolabeled substrate. Two g of liver from each animal was homogenized in 5 ml of the homogenizing buffer. Fifty μl of liver homogenate was used in each assay tube in a final assay volume of 200 μl. In this type of ex vivo experiment the effect of drug on liver ACAT can be measured under conditions in which loss of drug due to washing (i.e., microsome preparation) is minimized. In this same experiment changes in liver ACAT activity were compared to the concentration of cholesterol in both liver and plasma, and to the plasma apoB concentration, as determined by an immunoturbidimetric assay (20) using the Abbott VP Analyzer. Specific polyclonal antibody to rat apoB was raised in rabbits for this assay.

Rats treated with ethinyl estradiol

To assess whether orally administered CI-976 reaches the liver in an unchanged active form (or active metabolites), rats were dosed with CI-976 for 5 days while 17α-ethinyl estradiol was simultaneously administered subcutaneously (5 mg/kg per day in PEG-200–ethanol 95:5). Such pharmacologic doses of ethinyl estradiol markedly elevate LDL receptor activity (21), resulting in a profound hypolipidemia (22). The lipoprotein-cholesterol taken up by the liver is then esterified by ACAT and stored (23).

Since there is essentially no cholesterol present in the diet or plasma of these animals, any decrease in liver esters must occur independent of changes in exogenous cholesterol absorption or hypocholesterolemic action of the drug, and most likely involves a direct effect liver ACAT.

Sucrose-fed rats

In order to stimulate liver VLDL synthesis, rats were fed the purified sucrose diet described by Strobl et al. (24) except that the brewer's yeast was replaced with adequate vitamin supplements (formulated by Dr. E. Ulman, Research Diets, Inc., New Brunswick, NJ). CI-976 was dosed by daily gavage or by subcutaneous injection (using the same vehicles as described for cholesterol-fed rats). Drug and diet were started on the same day and continued for 11 days. The last dose was 18–24 h before sacrifice. Plasma triglycerides (16) and apoB (20) were measured as efficacy parameters in this model.

Biliary lipid and bile acid secretion

Rats fed chow, the PCC diet, or the PCC diet plus CI-976 (0.05% in the diet) for varying time periods were anesthetized with tiletamine/zolazepam (Telazol, 40 mg/kg) at 9 AM. The bile duct was cannulated with polyethylene-50 tubing as near to the ampulla as possible. The tubing was secured with sutures and cyanoacrylate ester (Super Glue, Loctite Corp.). The rats were placed on a heating pad set at 37°C during bile collection. Biliary drainage for the first 10 min was not collected; thereafter, bile was collected for 2 h (25). Bile lipids were measured by high performance liquid chromatography (HPLC) (see below), and total bile acids were determined using the 3α-hydroxy steroid dehydrogenase assay (26). As positive controls, we also evaluated the effects of cholic acid (1%), β-estradiol (5 mg/kg per day SC), cholestyramine (5% for 3 days), and diosgenin (1% for 1 week) in chow-fed rats.

Whole-body autoradiographic determination of the tissue distribution of ^{14}C-labeled CI-976

Another approach used to provide evidence for hepatic ACAT inhibition involved the use of whole-body autoradiography (27). Male and female Sprague-Dawley rats were dosed daily for 21 days with 50 mg/kg (50 μCi/kg), ^{14}C-labeled CI-976. Rats were killed at multiple time points postdose in duplicate by halothane anesthesia, and rapidly frozen in a dry ice/hexane bath. Specimens were embedded in methylcellulose ice and 50-μM sections were cut in a cryostat. Sections were allowed to air-dry at −20°C and attached to X-ray film for autoradiographic exposure periods which ranged from 4 to 12 weeks. Carbon-14 standards (American Radiolabeled Chemicals, St. Louis, MO) were included on representative films for assessment of processing consistency and calibration of the Loats 1024 Whole Body Autoradiography Image Analyzer (Loats Associates, Westminster, MD). Processed films were digitized with the analyzer scanner and radioactivity remaining in various tissues was determined by quantitative video densitometric analysis of digital images. Total radioactivity of the parent and radioactive metabolites remaining in the various tissues is expressed as μg-radioequivalents/g tissue.

Cholesterol absorption

Cholesterol absorption was determined in one series of experiments using the Zilversmit dual-isotope technique (28) according to the drug protocol described by Cayen and Dvornik (29). Briefly, rats were fed the PCC diet with or without (controls) drug for 1 week. On the last day, food was removed at 8 AM and the isotopes were administered beginning at 2 PM. [^{3}H]cholesterol (13 μCi/rat) was given by oral gavage and [^{14}C]cholesterol (1.5 μCi/rat) was given by tail vein injection. The [^{3}H]cholesterol was in the form of an emulsion, prepared by dissolving 125 mg cholesterol in 1625 mg olive oil. The oil phase was then suspended by sonication in 25 ml of water containing 156 mg taurocholate (sodium salt). Each animal received 1 ml. The intravenous dose was prepared by drying down the labeled cholesterol (50 μCi), and then adding 300 μl warm ethanol followed by 12.5 ml of saline. Each animal received 0.5 ml of this colloidal suspension. The rats were allowed to consume their respective diets at 3 PM, and were killed 48 h after isotope administration. The percentage of an oral dose of cholesterol absorbed was calculated from the plasma isotope ratio (% of the oral dose in 2 ml plasma/% of the intravenous dose in 2 ml plasma × 100).

More direct evidence for an inhibitory effect on cholesterol absorption was obtained using the lymph-fistula model. This model also provides an indication as to the duration of inhibition and the relative selectivity of the compound for the absorption of cholesterol versus triglyceride and phospholipid. Rats were anesthetized at 1 PM by an intramuscular injection of tiletamine/zolazepam (Telazol, 40 mg/kg). Silicone rubber cannulas were placed into the main mesenteric lymph duct (0.03" I.D. × 0.065" O.D.) and the duodenum (0.025" I.D. × 0.047" O.D.) and secured with sutures, as previously described (30). Animals were allowed to recover from the surgery overnight in restraining cages while infused intraduodenally with 2% dextrose in saline containing 0.03% KCl (2.5 ml/h). Drinking water was allowed ad libitum during this recovery period. At 6 AM the following day the drinking water was removed and a 2-h basal lymph sample was collected. At this point two different series of experiments were performed. In one series (bolus method), animals were given one of three ACAT inhibitors at a specified dose as a single bolus into the duodenal cannula using the aqueous CMC/Tween suspension vehi-

cle. Controls received a bolus injection of vehicle alone. Immediately after the drug dose, a lipid emulsion containing 0.1% cholesterol, 0.11% sodium taurocholate, 15% Intralipid (20%, Kabivitrium, Inc., Alameda, CA), 2.4% safflower oil, and 82.6% saline was infused into the duodenal cannula (3 ml/h). Then four additional 2-h lymph collections were obtained. In the other series of experiments (drug infusion method), CI-976 was continuously infused as part of the lipid emulsion at the rate of 1 mg/h. The lymph samples from the rats receiving bolus injection were extracted into hexane in the presence of a stigmasterol internal standard, and total (base hydrolyzed) and free cholesterol were quantitated by FID gas–liquid chromatography on a 6-ft column of OV-17 in a Varian Model 3700 GC equipped with a Varian Series 7000 autoinjector and a Hewlett-Packard 3390 Integrator. Esterified cholesterol content of lymph was determined by difference. In the drug infusion series of experiments, lymph lipids were determined by HPLC (see below).

We also used the lymph fistula model to examine the effect of CI-976 on the absorption of endogenous (i.e., biliary) cholesterol. In these experiments, cannulated rats were infused with the saline/dextrose solution to which 2% whole rat bile was added containing [^{14}C]cholesterol. No nonradiolabeled lipid other than that in bile was infused into these animals, and hence, lymph cholesterol was exclusively of biliary origin. Hourly collections of lymph were obtained with the use of fraction collectors. Lymph was extracted with 3 volumes of ethylacetate–acetone 2:1 (31). The total ^{14}C label in an aliquot of lymph extract was determined by liquid scintillation spectroscopy. The lymph extracts were resuspended in 100 μl chloroform–methanol 2:1 and spotted on Whatman LK6D silica TLC plates (Whatman, Hillsboro, OR). The plates were developed in hexane–diethylether–acetic acid 85:15:1. The distribution of ^{14}C label between the cholesteryl ester and cholesterol bands was visualized and quantified by exposing the plates (wrapped in plastic wrap) to storage phosphor imaging plates (Molecular Dynamics, Sunnyvale, CA) for 16 h and then scanning the imaging screens on a Molecular Dynamics Phosphorimager.

Lipid mass detection by HPLC

Lipids in aqueous samples were extracted with 3 volumes of ethylacetate–acetone 2:1 according to the procedure of Slayback, Cheung, and Geyer (31). Pregnenolone was added to the extraction mixture to serve as an internal standard for the mass detector. The lipids were resuspended in isooctane–tetrahydrofuran 99:1 and chromatographed on Spherisorb, 10 cm × 4.6 mm, 5 μm silica, Phase-Sep column (Norwalk, CT) placed in a 40°C column oven. The HPLC system consisted of a Spectra-Physics 8800 Ternary Gradient Pump and Spectra-Physics 8880 Autosampler controlled by SpectraSTATION software (Spectra-Physics, Santa Clara, CA) according to the chromatographic conditions published by Christie (32), with the exception that methylene chloride was used in place of chloroform. The masses of eluted lipids were detected with a Varex evaporative light scattering detector (ELSD II) equipped with a Varex Linearizer (Varex, Rockville, MD). The nitrogen flow was set at 50 mm and the drift tube temperature was 125°C. The detector response was calibrated by chromatographing lipid mixtures containing known quantities of each of the lipids of interest.

RESULTS

In vitro potency and selectivity

In the in vitro assay system using rabbit intestinal microsomes, ACAT activities were in the range of 1300 to 1800 pmol/mg per min. With respect to enzyme inhibition, CI-976 and SaH 58-035 were approximately equal in potency (IC_{50} = 0.073 and 0.057, respectively). In contrast, Bay o 2752, SaH 57-118, and octimibate possessed activities in the micromolar range (4.4, 5.8, and 30 μM, respectively). Potencies of melinamide and CL 277,082 were intermediate between these compounds (0.45 and 0.14 μM, respectively). Thus, CI-976 was approximately twofold more potent than CL 277,082. Three compounds were also evaluated using microsomes from livers of cholesterol-fed rats. Control activity was 74 pmol/min per mg. CI-976 and SaH 58-035 were somewhat less potent in rat liver compared to rabbit intestine (0.18 and 0.11 μM, respectively) while CL 277,082 was about threefold less potent (0.47 μM). Our observed IC_{50} values for compounds other than CI-976 are in general agreement with published values (3, 6, 7, 33–37) despite the slight differences among assays.

CI-976 up to 100 μM had no in vitro effect on rat intestinal CEH. This has subsequently been confirmed assaying ester hydrolysis with the bovine pancreatic enzyme (data not shown). Some inhibition of MGAT (45%) and LCAT (62%) was observed, however, at 100 μM, a concentration > 1000-fold that required to inhibit ACAT. Because the peak plasma drug concentration in rats after daily oral gavage for 2 weeks ranges from 2 to 12 μM (50 to 400 mg/kg dose range; A. Black and T. Woolf, unpublished results), it is highly unlikely, based on these data using the rat enzyme, that plasma LCAT would be inhibited by CI-976 in vivo in this species. But the range of concentrations of drug within intestinal cells is presently unknown, and it is conceivable that intestinal MGAT activity could be partially inhibited in vivo at the site of drug absorption. In these specificity assays the CEH inhibitor inhibited activity by 97% at 100 μM (15) and DTNB inhibited LCAT by > 99% at 1.176 μM (14), respectively. CL 277,082, SaH 58-035, and melinamide also had no

effect on LCAT or MGAT up to 100 μM (data not shown), thus confirming previous reports (1, 6, 7). It has also been demonstrated that CI-976 has no effect on retinol esterification using the CaCo-2 cell model (38).

Efficacy in cholesterol-fed rats

In normal chow-fed rats, chronic treatment with CI-976 lowers plasma triglycerides but not plasma cholesterol (data not shown). Therefore, chronic efficacy (i.e., cholesterol lowering activity) was studied in cholesterol-fed rats (PCC diet). In one series of chronic experiments, rats were fed the PCC diet for 2 weeks and dosed with ACAT inhibitors during the second week. In other words, the compounds were administered to animals with preestablished dyslipidemia (high nonHDL-cholesterol, low HDL-cholesterol). Five different ACAT inhibitors were initially evaluated (**Fig. 2**). Bay o 2752 was inactive, and melinamide only lowered plasma nonHDL-cholesterol at the highest dose of 100 mg/kg (−42%). Reductions for octimibate were significant at 10 and 100 mg/kg, but not at 30 mg/kg. CI-976 was more potent than CL 277,082. CI-976 significantly reduced cholesterol by 36% at 10 mg/kg but the decrease due to CL 277,082 (−16%) did not reach significance. However, at higher doses (30 and 100 mg/kg) both CI-976 and CL 277,082 gave similar reductions in nonHDL-cholesterol. The calculated ED_{50} values (i.e., doses required to lower nonHDL-C by 50%) for these inhibitors were identical (19 mg/kg).

All ACAT inhibitors evaluated increased the low HDL-cholesterol concentrations (15 mg/dl) typically observed in this cholesterol-fed model. At 10 mg/kg the response to octimibate (+47%) was significantly different from both control values and from the CL 277,082 group (−13%). Other inhibitors were inactive at this dose, but at 30 mg/kg both octimibate and CI-976 significantly elevated HDL-cholesterol. At the highest dose (100 mg/kg) all compounds significantly elevated HDL-cholesterol, but only CI-976 elevated it to the level found in normal chow-fed rats (49 mg/dl). Thus, in terms of increases in HDL-cholesterol, CI-976 was the most efficacious of the inhibitors examined.

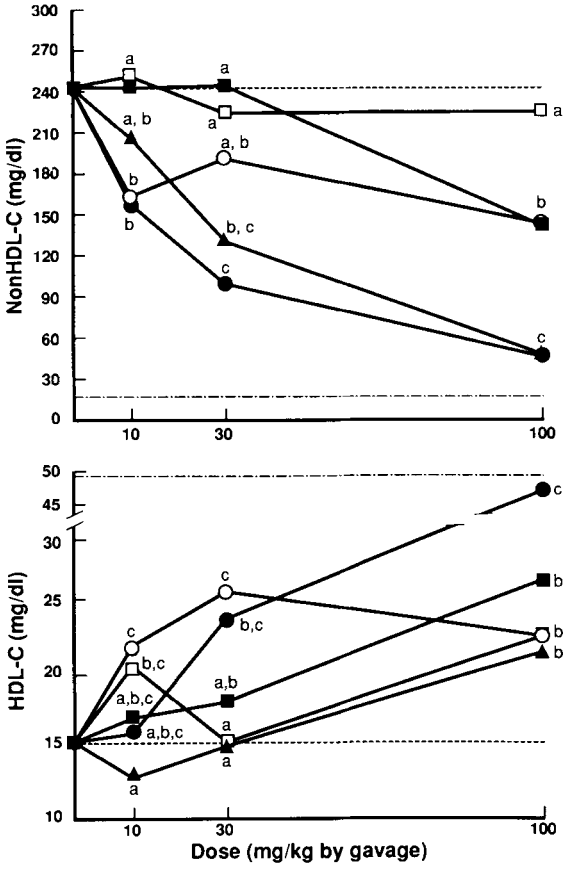

Fig. 2. Dose-response data for ACAT inhibitors in the chronic cholesterol-fed rat model. Decreases in nonHDL-cholesterol (top panel) and increases in HDL-cholesterol (bottom panel) are indicated. Rats were fed the PCC diet for 2 weeks and dosed by daily gavage with the drugs during the second week only. In PCC controls the nonHDL-cholesterol and HDL-cholesterol values were 243 mg/dl and 15 mg/dl, respectively (------). Corresponding values for rats switched to normal chow during the second week are also indicated (-•--•-). Data points are the means of six animals/group. Values with the same superscripts are not significantly different ($P < 0.05$) based on ANOVA followed by a protected least-significant difference test. Values with an "a" superscript are not different from PCC values. (●) CI-976, (○) octimibate, (■) melinamide, (□) Bay o 2572, (▲) CL 277,082.

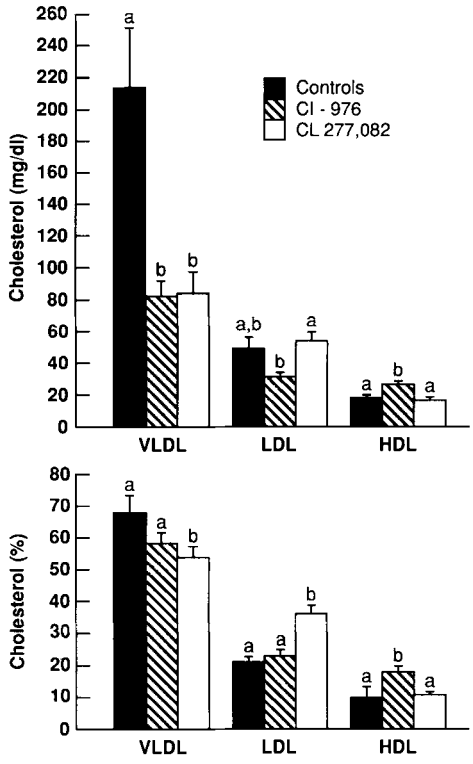

Fig. 3. Effect of CI-976 and CL 277,082 on cholesterol distribution as determined by HPGC (18). Data are expressed in absolute terms (mg/dl, top panel) and as a percentage of total cholesterol (bottom panel). For each lipoprotein class, bars with different superscripts are significantly different ($P < 0.05$).

TABLE 1. Effect of ACAT inhibitors on liver cholesteryl ester concentration in the chronic cholesterol-fed rat model

Compound	3	10	30	100	Control Value
		% – changes			mg/g
CI-976	– 9	– 37	– 43	– 74	14.6 ± 1.1
Octimibate	– 26	– 4	+ 17	– 40	14.6 ± 1.1
Melinamide	– 17	– 9	– 15	– 4	14.6 ± 1.1
CL 277,082	– 13	– 21	– 26	– 15	22.2 ± 3.3
Bay o 2752	– 8	– 23	– 45	– 45	22.7 ± 0.7
SaH 58-035	– 25	– 8	– 20	– 29	22.7 ± 0.7

Values are the percent changes from control rats fed the PCC diet. Three separate experiments are shown, each with a separate control group (n = 6/dose group).

As an index of overall changes in cholesterol distribution, we calculated the ratio of HDL-cholesterol to nonHDL-cholesterol (× 100), termed the antiatherosclerotic index by others (39). CI-976 produced the greatest increase in this index, from a value of 6 in PCC controls to values of 10, 24, and 98 at dose levels of 10, 30, and 100 mg/kg, respectively. Values for the other inhibitors were significantly lower at 30 and 100 mg/kg (data not shown).

As a reference it should be noted that beta-sitosterol was also active in this rat model, but at much higher doses. At 6% (w/w) in the diet, it lowered nonHDL-cholesterol about 65% and elevated HDL-cholesterol about 20%. In a separate experiment we also evaluated SaH 58-035 at the same doses (10, 30, and 100 mg/kg) and found it to be inactive.

Since the changes in cholesterol distribution noted above in the chronic rat model were obtained by a precipitation method, we decided to confirm these changes in the distribution of cholesterol in the same plasma samples using Superose 6HR-HPGC (18). We compared the PCC controls to the groups receiving the two inhibitors that induced the greatest changes in nonHDL-cholesterol, i.e., CI-976 and CL 277,082. In addition, we chose the intermediate dose of 30 mg/kg for which responses to these inhibitors were similar for nonHDL-cholesterol but different for HDL-cholesterol using the precipitation method. **Fig. 3** illustrates that in PCC controls almost 70% of the total cholesterol resides in particles the size of VLDL, while the remainder is in LDL (21%) and HDL (10%). The values for HDL-cholesterol obtained by precipitation and HPGC were similar (14 and 16 mg/dl, respectively). As found using precipitation, CI-976 but not CL 277,082 elevated HDL-cholesterol at this dose. The HPGC analysis also revealed differences in VLDL and LDL cholesterol between groups. The LDL-cholesterol values for CI-976 were significantly lower than those for CL 277,082, even though these drugs lowered VLDL-cholesterol to the same extent. In fact, the changes were in the opposite direction: CI-976 lowered LDL-cholesterol by 35%, while CL 277,082 elevated LDL-cholesterol by 10%.

All ACAT inhibitors decreased liver cholesteryl ester concentrations in rats with preestablished dyslipidemia, and these data are presented in **Table 1.** In all experiments cholesteryl esters accounted for greater than 70% of the total cholesterol. CI-976 produced a dose-dependent decrease in liver cholesteryl ester concentration up to 74% at the highest dose (100 mg/kg). Bay o 2752 decreased liver esters up to 45%. In contrast, the changes due to the other compounds were of lesser magnitude and not clearly related to dose. All of the compounds tended to decrease liver unesterified cholesterol, therefore, in no case did this substrate for ACAT accumulate in the livers of treated animals (data not shown).

Cholesterol-fed rats: parenteral versus oral dosing

The prevention of diet-induced dyslipidemia in rats by CI-976 was demonstrated by both oral and parenteral routes of drug administration. As shown in **Fig. 4,** CI-976 was also equally effective in altering plasma cholesterol concentration and distribution when administered by subcutaneous injection. In fact, at the highest dose tested (30 mg/kg), efficacy (nonHDL-C lowering, HDL-C elevation) was greater with the SC route compared to the oral route. In a more recent experiment we have observed that CI-976 administered SC also lowers plasma cholesterol in normal chow-fed rats after 2 weeks of treatment (14% and 28% at 30 and 100 mg/kg, respectively).

Cholesterol-fed rats: sex differences

Male and female rats of similar body weight (151–161 g) were fed the PCC diet for 1 week, and then given five daily oral doses of CI-976. Females tended to have higher nonHDL-cholesterol concentrations compared to males on the PCC diet, but the difference was not statistically different. Other differences were significant. For example, at 10 and 30 mg/kg per day, nonHDL-cholesterol concentration decreased 34% and 67% in males, but 49% and

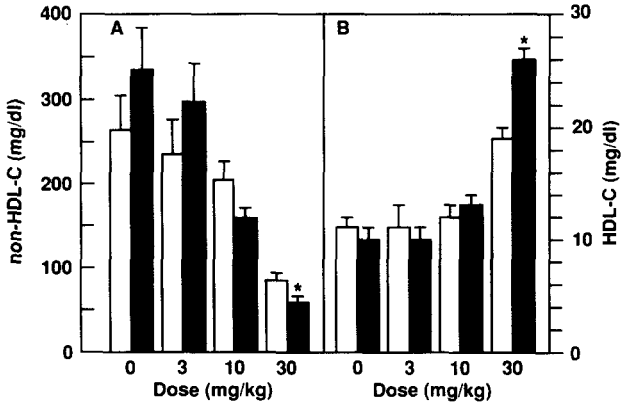

Fig. 4. Effect of CI-976 administered orally (open bars) or by subcutaneous injection (closed bars) on nonHDL-cholesterol (panel A) and HDL-cholesterol (panel B) in cholesterol-fed rats. The asterisk indicates a significant difference between drug routes ($P < 0.05$).

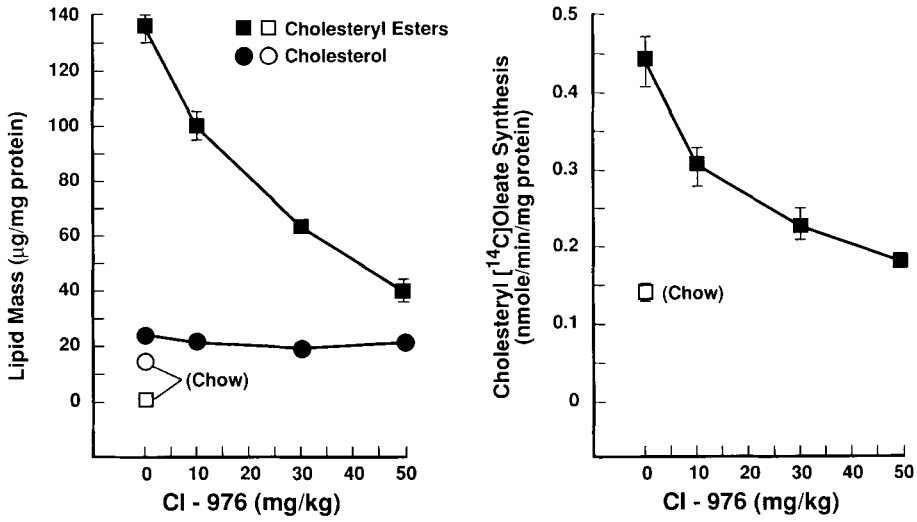

Fig. 5. Liver cholesterol and cholesteryl ester mass (left panel) and ACAT activity (right panel) in rats treated with CI-976. Rats were fed the PCC diet and dosed by daily gavage at the indicated doses for 5 days. Approximately 18 h after the last dose the livers were removed, and homogenates were prepared for measuring ACAT activity. An aliquot was also used to determine cholesterol and cholesteryl ester content by HPLC (see Methods). Open symbols indicate values observed in chow-fed rats for comparison. Values are the mean ± SEM for six rats/group.

83% in females, respectively. HDL-cholesterol was 15 mg/dl in males and only 6 mg/dl in females. In males, HDL-cholesterol increased only at the 30-mg/kg dose (+61%), but in females HDL-cholesterol increased 173% and 600% at 10 mg/kg and 30 mg/kg, respectively. Therefore, female rats hyperresponded to the PCC diet (lower HDL) and to CI-976 treatment.

Cholesterol-fed rats: ex vivo ACAT activity

CI-976 was administered by oral gavage for 5 days while animals consumed the PCC diet. Eighteen hours after the last dose the rats were killed and livers were removed for measurement of ACAT activity in freshly prepared homogenates. Under these conditions, ACAT activity was threefold higher in PCC controls compared to chow-fed rats, and declined exponentially with increasing dose of CI-976 (**Fig. 5**). At 50 mg/kg ACAT activity was reduced 90%. Accompanying these changes in liver ACAT were dose-related decreases in cholesteryl ester mass. Free cholesterol and phospholipid mass did not change. Liver triglyceride increased 360% in PCC controls compared to chow-fed rats, but CI-976 did not significantly decrease liver triglyceride (data not shown). Plasma cholesterol was decreased from 55% (10 mg/kg) to 78% (50 mg/kg) and plasma apoB decreased from 29% (10 mg/kg) to 59% (50 mg/kg) in this experiment.

17α-Ethinyl estradiol-treated rats

As anticipated, 17α-ethinyl estradiol treatment caused a marked hypolipidemia, resulting in plasma cholesterol values that were less than 10 mg/dl in all treatment groups. As a result of this accelerated removal of plasma cholesterol, the liver cholesteryl ester concentration increased significantly, even though the animals were consuming a chow diet (**Fig. 6**). Orally administered CI-976 partially prevented this accumulation of esters at 30 mg/kg, and completely restored ester levels to normal at 150 mg/kg. Free cholesterol concentration in the liver increased significantly at these same doses.

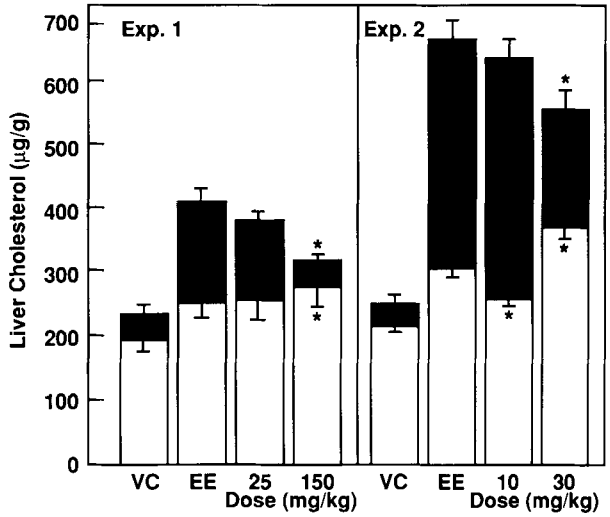

Fig. 6. Liver cholesterol concentration in vehicle control (VC) and ethinyl estradiol-treated (EE) rats treated with CI-976. Rats were injected daily with EE by SC injection and with CI-976 by daily gavage at the indicated doses. Two separate experiments are depicted. Shaded areas represent cholesteryl ester mass, open areas are free cholesterol. Values are means SEM for seven animals/group. *, Significantly different from EE controls ($P < 0.05$) for free (bottom) or esterified (top) cholesterol.

TABLE 2. Effect of CI-976 in sucrose-fed rats

CI-976 Treatment Dose	Route	Plasma Cholesterol	Plasma Triglycerides	Plasma Triglycerides	Plasma HDL-Cholesterol
mg/kg		*mg/dl*	*mg/dl*	*% – change*	*mg/dl*
0 control	oral	73 ± 4	643 ± 66		50 ± 4
3	oral	80 ± 6	427 ± 40[a]	– 34	55 ± 4
10	oral	72 ± 4	332 ± 33[a]	– 48	56 ± 4
30	oral	83 ± 6	362 ± 48[a]	– 44	63 ± 5[c]
100	oral	73 ± 4	299 ± 29[a,b]	– 53	58 ± 3
0 control	SC	75 ± 4	442 ± 41		51 ± 4
3	SC	70 ± 3	273 ± 29[a]	– 38	53 ± 3
10	SC	66 ± 5	193 ± 11[a,b]	– 56	58 ± 5
30	SC	62 ± 4	216 ± 33[a]	– 51	64 ± 5[c]
100	SC	55 ± 5	150 ± 19[a,b]	– 66	64 ± 6[c]

Values are the mean ± SEM (n = 7/group); SC, subcutaneous.
[a] Significantly different from control using the same drug route ($P < 0.05$).
[b] Significantly different from 3 mg/kg dose group ($P < 0.05$).
[c] Marginally significant from control ($P < 0.10$).

Sucrose-fed rats

CI-976 significantly reduced plasma triglycerides in rats with sucrose-induced hypertriglyceridemia at all doses examined (3–100 mg/kg dose range, **Table 2**). Thus, at the present time, the lowest significant dose remains to be established using this model. Triglyceride lowering was observed with both oral and SC routes, the latter being somewhat more effective on a percentage basis at doses greater than 3 mg/kg even though the SC control values were lower compared to oral controls. In a separate experiment oral dosing resulted in similar dose-related decreases in triglycerides, but plasma apoB decreased by about 20% in all dose groups to values found in chow-fed rats.

Biliary lipids and bile acids

Compared to normal chow-fed rats, we found that rats fed the PCC diet secrete more bile acids (+78%), cholesterol (+18%), and phospholipid (+79%). Bile acid secretion was further increased almost 30% in PCC rats treated with CI-976 (50 mg/kg) for 1 week (**Table 3**). This effect declined to about 20% after 2 weeks. CI-976 also increased cholesterol secretion, but in contrast to bile acids, the stimulatory effect of CI-976 was greater after 2 weeks (+77%) compared to 1 week (+30%). Biliary phospholipid secretion paralleled the changes in cholesterol, i.e., increased at 2 weeks (+53%), and little change (+20%) after 1 week. To verify this animal model, we evaluated four reference agents in normal chow-fed rats. Feeding cholic acid to rats resulted in the expected (40) increases in bile acids (61%), cholesterol (96%), and phospholipid (107%). Therefore, the differences between the chow-fed and PCC-fed rats noted above may be due largely to the dietary cholic acid. The SC injection of ethinyl estradiol decreased biliary bile acid secretion (40%), as reported by Davis and Kern (41), and cholestyramine (diet-admix) decreased bile acid (82%)

TABLE 3. Effect of CI-976 on biliary lipid and bile acid secretion in the rat

Treatment[a]	Duration	n	Bile Acids	Cholesterol	Phospholipid
	weeks			*μmol/kg/hr*	
Normal chow	1	15	122.1 ± 12.4	2.0 ± 0.2	8.3 ± 1.1
PCC diet	1	8	217.9 ± 29.2	2.3 ± 0.3	14.5 ± 1.9
PCC diet	2	9	219.3 ± 28.7	2.6 ± 0.6	15.6 ± 3.0
PCC + CI-976	1	10	280.6 ± 10.1[b] (+29%)	3.0 ± 0.1[c] (+30%)	17.4 ± 2.0 (+20%)
PCC + CI-976	2	5	265.0 ± 33.2 (+21%)	4.6 ± 1.1[c] (+77%)	23.6 ± 2.1[c] (+51%)

Values are the mean ± SEM.
[a] Bile flow (mL/hr) was not different among treatment groups.
[b] Significant from PCC controls at 1 week, $P < 0.05$.
[c] Marginally significant from respective PCC controls, $P < 0.10$.

and phospholipid (77%) output without significantly altering biliary cholesterol (42). Finally, diosgenin (dietadmix) increased biliary cholesterol secretion threefold (25) without changing bile acid or phospholipid output. These confirmatory data for reference agents help support the validity of the data for CI-976.

Whole-body autoradiography

The distribution of ^{14}C-labeled CI-976 radioequivalents in tissues was examined by whole-body autoradiographic techniques in both male and female rats after chronic dosing. In male rats at 24 h after the last dose very high radioactivity was found in the caecum, large intestine, and rectal contents. Somewhat lower but still substantial radioactivity was present in the contents of the small intestine and stomach. High activity was noted in bile ducts, urinary bladder, ureters, and renal pelvis. As radioactivity in these regions is due primarily to unabsorbed drug or drug elimination, the data are not included in the table. Among the other sites of deposition, liver and body fat were the most highly labeled tissues, considerably higher than adrenal, brown fat, and thyroid gland (**Table 4**). Relatively low radioactivity remained in other tissues, including heart, blood, testis, and spleen. Activity in female rats at 24 h was in general higher than in males. In particular, body fat and liver radioactivities were higher, suggesting the possibility of sex-related differences in drug metabolism as observed above for efficacy in cholesterol-fed rats. Radioactivity persisted at 120 h with highest values occurring in the distal GI tract with only minor traces in stomach contents. Among other sites liver and body fat retained the highest radioactivity, suggesting a long half-life in these tissues.

TABLE 4. ^{14}C-labeled CI-976 distribution in rat tissue after chronic dosing

	Hours after Last Dose			
	24 h		120 h	
Tissue	Male	Female	Male	Female
		μg/g		
Adrenal	4.72	11.05	BLQ	BLQ
Blood	BLQ	2.05	BLQ	BLQ
Brown fat	2.77	7.49	BLQ	BLQ
Fat	11.29	32.47	1.55	2.84
Harder's	2.69	7.76	BLQ	BLQ
Heart	BLQ	2.83	BLQ	BLQ
Kidney	3.57	7.42	1.40	BLQ
Liver	14.89	17.64	4.71	2.14
Lung	BLQ	2.15	BLQ	BLQ
Salivary	0.70	2.69	BLQ	BLQ
Spleen	1.15	2.33	BLQ	BLQ
Thyroid	2.08	2.74	BLQ	BLQ

BLQ, below limit of quantitation; 1.06 μg/g.

Cholesterol absorption

A single bolus of CI-976 significantly blunted the increase in the lymphatic transport of cholesteryl esters in rats infused with lipid emulsion using the lymph-fistula model (**Fig. 7**). The dose required for a significant effect under these conditions was 30 mg/kg, and these changes occurred at 4–6 h (−41%), 6–8 hours (−42%), and 8–10 h (−48%) after drug administration. Therefore, the duration of action was at least 8 h. The changes at 100 mg/kg were similar to those at 30 mg/kg (40–50% decreases), suggesting that a maximal effect had been reached under these conditions with CI-976. CL 277,082 and SaH 58-035 were inactive in this model, although SaH 58-035 tended to decrease cholesteryl ester transport at the highest dose (25–30% changes). None of the drugs significantly altered the transport of lymph unesterified cholesterol, triglycerides, and phospholipid in these experiments (data not shown). The lymph triglyceride data support the lack of effect of CI-976 on rat intestinal MGAT activity.

When CI-976 was continuously infused into the duodenal cannula as part of the lipid emulsion, the effect on cholesteryl ester transport was more marked, with greater than 80% reductions in lymph cholesteryl ester at the later time points (**Fig. 8**). In these drug infusion experiments, the transport of both triglyceride and phospholipid tended to be lower, and the transport of unesterified cholesterol tended to be higher in the CI-976 groups after 8 h of infusion, but these differences were not statistically significant (data not shown).

Inhibition of cholesterol absorption by CI-976 was also demonstrated in rats by the dual-isotope method. In these experiments the drug was mixed into the food for 1 week prior to the isotope administration. Animals fed the PCC diet tended to absorb a slightly greater percentage of cholesterol compared to chow-fed controls (62% vs. 57%). Chronic treatment with CI-976 by diet admix inhibited cholesterol absorption by 44%, 48%, and 66% at doses of 12.5, 50, and 100 mg/kg, respectively. Dose-response data was not obtained for the other ACAT inhibitors.

We also used the lymph-fistula model to obtain information on the absorption of endogenous or biliary cholesterol (Fig. 9). This was done by infusing 2% rat bile, labeled with [^{14}C]cholesterol, into the duodenal cannula. Lymph radioactivity was then monitored as a function of time. CI-976 (30 mg/kg) was injected into the duodenum 11 h after the initiation of isotope infusion as it was determined that this represented the midpoint on the linear curve for [^{14}C]cholesteryl ester output, and any change in this rate would be easily detected. This, in fact, occurred after CI-976 injection. Radiolabeled ester output decreased to almost zero within 2–3 h, and then slowly returned to control (vehicle) levels by 22–23 h. The duration of action appeared to be similar to that for the

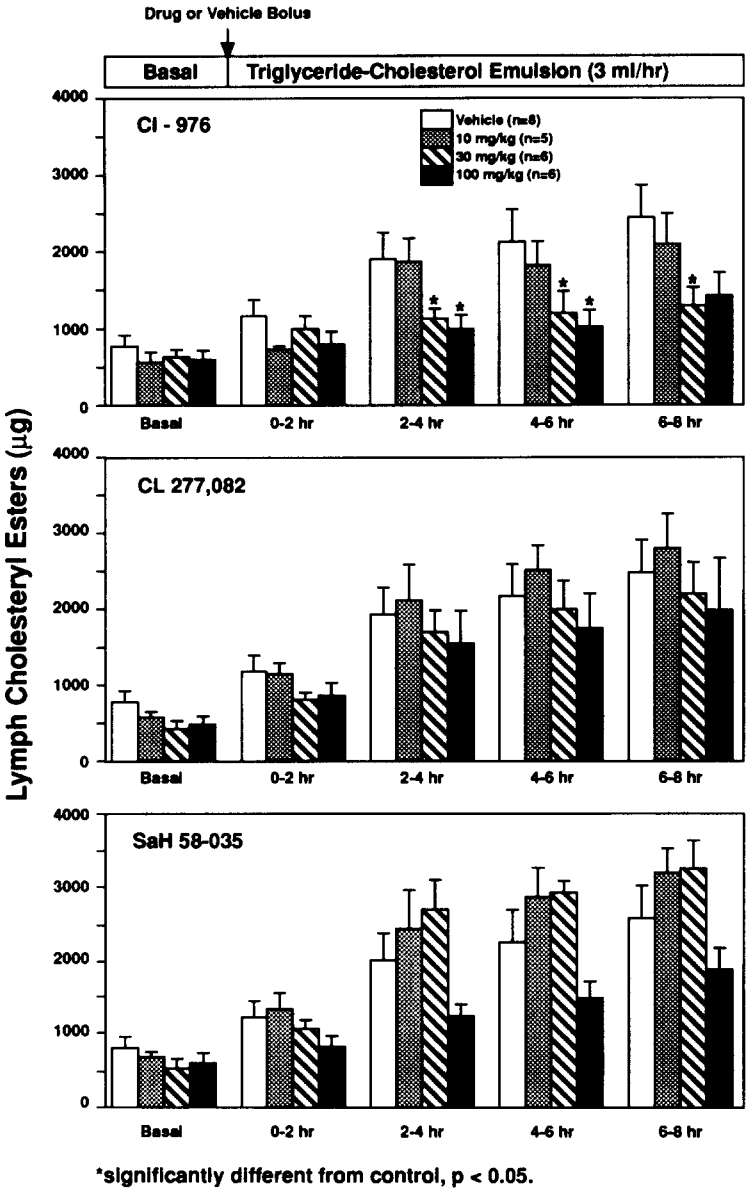

Fig. 7. Effect of a single bolus of ACAT inhibitor on the subsequent appearance of cholesteryl ester in mesenteric lymph. Basal lymph was collected for 2 h, and then a single dose of drug or vehicle alone (controls) was injected into the duodenal cannula. The duodenal lipid infusion was then started and lymph was collected at 2-h intervals. *, Significantly less than controls at the same time point ($P < 0.05$).

inhibition of exogenous cholesterol absorption, i.e., 6–8 h (see Fig. 8). The output of radiolabeled free cholesterol was also transiently decreased by a single dose of CI-976.

DISCUSSION

CI-976 is a potent inhibitor of ACAT in microsomes obtained from the intestine and liver of cholesterol-fed animals. Among the ACAT inhibitors described in this study, it is more potent in vitro than all except SaH 58-035. It has been established that SaH 57-118 (6), melinamide (33), CL 277,082 (7), and Bay o 2752 (3) do not inhibit the other cholesterol esterifying enzyme thought by some (20) to be involved in cholesterol absorption, namely, pancreatic cholesterol esterase. We have found this to be true for CI-976 as well, and also have determined specificity with respect to MGAT, LCAT, and ARAT (38). This type of enzyme characterization or profiling is essential before statements can be made regarding in vivo consequences of ACAT inhibition with CI-976 treatment.

CI-976, like melinamide (43) and CL 277,082 (7), lowers plasma and liver cholesterol concentrations in cholesterol-fed rats. In the present study this was illustrated after multiple doses in a chronic model of dyslipidemia. An aqueous vehicle was used for gavage administration of compounds. The apparent differences in potency and efficacy among the inhibitors tested should therefore be interpreted with the knowledge that absorption may

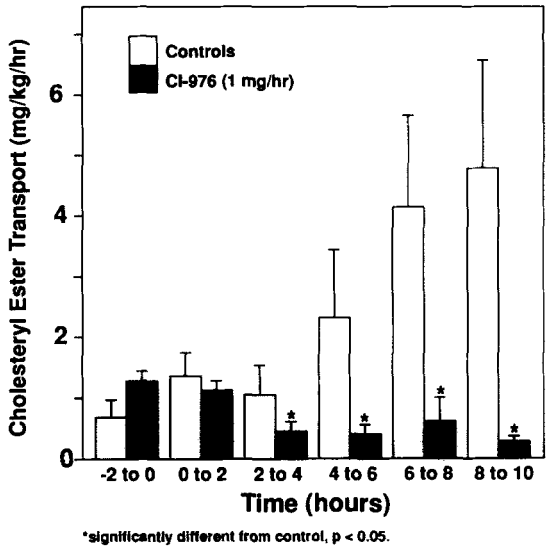

Fig. 8. Effect of the infusion of CI-976 into the duodenum on the mesenteric lymphatic output of cholesteryl esters. After the collection of basal lymph for 2 h, rats were infused with lipid emulsion or lipid emulsion containing CI-976. The rate of drug infusion was 1 mg/h (4 to 5 mg/kg per h). *, Significantly different from controls at the same time point ($P < 0.05$).

not be optimal under these conditions for each compound. In this rat efficacy model HDL-cholesterol was elevated, especially in female rats due possibly to sex-related differences in drug metabolism. At the present time the primary mechanism for the increase in HDL-cholesterol in cholesterol-fed rats (in the absence of plasma triglyceride lowering) for CI-976 is unknown. It is possible that HDL elevation is simply a reflection of the ability of CI-976 to inhibit cholesterol absorption, as dietary cholesterol decreases HDL-cholesterol and removal of dietary cholesterol increases HDL-cholesterol in this animal model. This cannot be the only mechanism, however, since the parenteral administration of CI-976 also results in an elevation of HDL-cholesterol, and, after SC administration, biliary excretion of the drug is probably not of sufficient magnitude to inhibit cholesterol absorption. Alternatively, HDL elevation by CI-976 (normalization) in cholesterol-fed rats may be associated with changes in apoE distribution as shown previously with gemfibrozil in cholesterol-fed rats (44).

In the present study several lines of evidence have been presented using rat models supporting the concept that CI-976 does in fact reach the liver to inhibit ACAT, and thereby alter hepatic lipoprotein production: *1)* CI-976 lowers plasma triglycerides in normal chow-fed rats. This could be due to accumulation of triglyceride in the intestine since Kam et al. (45) have shown that triglycerides accumulate in cultured CaCo-2 cells incubated with SaH 58-035. That this effect is not due to inhibition of triglyceride absorption, however, is illustrated by the mesenteric lymph experiments. Nonetheless, we cannot rule out a chronic effect on triglyceride absorption since only acute effects were studied, and lymph has not been collected from animals chronically treated with CI-976. *2)* CI-976 lowers plasma cholesterol when dosed subcutaneously in cholesterol-fed rats. Although this strongly suggests a hepatic effect, it is possible that SC-administered CI-976 reaches the intestinal mucosal cell to some extent. In fact, in separate experiments some radioactivity was found in the intestine after the IV injection of ^{14}C-labeled CI-976 (data not shown). Essentially, no unchanged drug would be expected to be present in the bile after SC injection, however, to inhibit ACAT from the lumenal side. At the very least, efficacy by the SC route could be due, at least in part, to inhibition of hepatic ACAT. *3)* CI-976 reduces the accumulation of cholesteryl esters in the livers of rats treated with ethinyl estradiol, a well-documented model of accelerated lipoprotein removal and hepatic ester storage. The relatively higher doses required compared to the other rat models may relate to absorption of

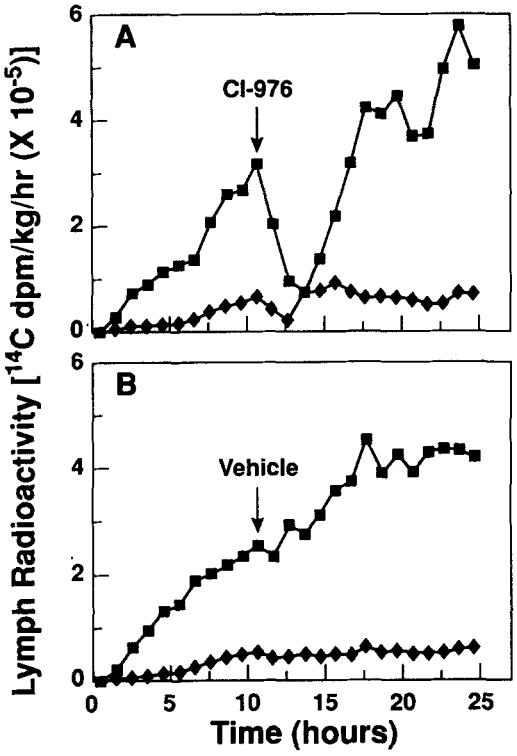

Fig. 9. CI-976 inhibition of biliary cholesterol absorption in lymph-fistula rats. A saline solution of 2% rat bile containing [^{14}C]cholesterol (0.05 μCi/ml) was infused into the duodenum at 2.5 ml/h. Intestinal lymph output was collected in 1-h fractions from a cannula implanted in the mesenteric lymphatic duct. At 11 h after the initiation of [^{14}C]cholesterol-labeled bile infusion, a volume (approximately 0.3 ml) of polyethyleneglycol (PEG) containing CI-976 (33.3 mg/ml) was infused into the duodenal cannula of the experimental animals to yield a CI-976 dose of 30 mg/kg (A). Control animals (B) received the equivalent volume of PEG alone. The total amounts of [^{14}C]cholesteryl ester (■) and [^{14}C]cholesterol (♦) in each lymph fraction were determined as described in Materials and Methods.

the drug using a normal chow diet. *4)* In rats with sucrose-induced, hepatic overproduction of VLDL, CI-976 lowers plasma triglycerides dose-dependently by both oral and SC routes. This suggests not only that the drug reaches the liver but also that ACAT plays a role in the assembly and/or secretion of VLDL-triglycerides during carbohydrate loading (see below). *5)* Chronic treatment with CI-976 lowers both liver cholesteryl ester mass and the apparent rates of cholesterol esterification (ACAT activity) in livers from treated rats (ex vivo data). The decrease in ex vivo liver ACAT activity was far greater (90% at 50 mg/kg dose) compared to that reported for CL 277,082 (21% at 100 mg/kg) (46), although in the latter study microsomes were prepared, and drug could have been washed out. But these differences in ex vivo liver ACAT data do agree with the IC_{50} values since CI-976 is about 2.5-fold more potent that CL 277,082 in rat liver microsomes. In addition to the ex vivo ACAT data, we observed no accumulation of free cholesterol under conditions in which cholesteryl ester accumulation was inhibited. Based on the liver cholesterol and bile data, it appears that the fraction of dietary cholesterol that is absorbed fails to accumulate in the liver, and instead is excreted in the bile either in the form of bile acids or cholesterol. This agrees with the observation by Nervi et al. (47) describing a negative correlation between biliary cholesterol output and hepatic ACAT activity and ester mass in the rat. It is also relevant that SaH 58-035 causes an increase in bile acid secretion in primary cultures of rat hepatocytes (48). *6)* Radiolabeled CI-976 accumulates in the liver after chronic dosing. This cumulative evidence suggests that efficacy (plasma lipid lowering) is due in part to inhibition of liver ACAT. Although similar data are not presented for all ACAT inhibitors, we would predict that potent inhibitors that reach the liver would have a similar pharmacologic profile.

The conclusion that liver ACAT in the cholesterol-fed rat is a major determinant of the plasma cholesterol concentration is supported by previous work using cultured rat hepatocytes. For example, Drevon, Engelhorn, and Steinberg (49) have shown that changes in ACAT activity in cultured rat hepatocytes can determine the cholesteryl ester content of secreted VLDL, and Davis, McNeal, and Moses (50) reported that in hepatocytes from cholesterol-fed rats, cholesteryl esters compete with triglyceride for incorporation into the core of VLDL particles. Thus, cholesteryl ester-rich VLDL are secreted from liver cells with high levels of ACAT and increased cholesteryl ester mass. It is also well known that perfused livers from cholesterol-fed rats secrete large amounts of cholesteryl esters in the form of VLDL and beta-migrating LDL (51). With respect to the triglyceride-lowering activity of CI-976 in sucrose-fed rats, Khan, Wilcox, and Heimberg (52) have demonstrated a positive correlation between the secretion of VLDL-triglyceride by perfused rat liver and the concentration of hepatic cholesteryl esters. They further postulate that cholesteryl esters themselves may regulate VLDL-triglyceride secretion (53). Moreover, we have recently shown that CI-976 decreases triglyceride secretion but not synthesis in HepG2 cells (R. Homan and B. R. Krause, unpublished data). Taken together, data from cholesterol-fed rats and sucrose-fed rats suggest that if ACAT is inhibited, the predominant core lipid secreted in VLDL decreases. The in vivo pharmacologic evidence further suggests that there is a link between liver ACAT, liver cholesteryl ester concentration, VLDL composition/secretion, and cholesterol (cholesterol-fed model) or triglyceride (sucrose-fed model) concentrations in plasma of rats. Furthermore, CI-976 decreased plasma apoB in both hypercholesterolemic and hypertriglyceridemic rat models, and therefore the possibility exists that our results reflect a regulatory role in vivo of ACAT on apoB secretion, as postulated in vitro using HepG2 cells (54).

Several recent observations suggest that the importance of liver ACAT in animals is not limited to the rat. CI-976 also lowers plasma total cholesterol, apoB, and LDL-cholesterol in rabbits fed a cholesterol-free purified diet (55), and monkey livers perfused with CI-976 secrete less apoB and cholesteryl ester (56). We have also observed significant reductions in plasma cholesterol after SC administration of CI-976 to cholesterol-fed hamsters (B. R. Krause, R. Bousley, and R. Stanfield, unpublished observation). It is therefore possible that liver ACAT directly or indirectly regulates hepatic lipoprotein secretion in a variety of animal species. Whether these observations have any relevance to the treatment of human lipid disorders is currently unknown. The range of specific activities of ACAT in the liver of cholesterol-fed rats that we and others (57, 58) have found (74–586 pmol/min per mg) overlaps with values for normal human liver (7–86 pmol/min per mg) (59–61). Whether these values in normolipidemic humans represent minimum values compared to dyslipidemic subjects is unknown. In studies with normal human subjects, elevated cholesteryl oleate to cholesteryl linoleate ratios in the $d < 1.019$ g/ml fraction have been described (62), suggesting a greater contribution of ACAT-derived esters in circulating hepatic lipoproteins.

Direct evidence for cholesterol absorption inhibition has been provided both by the dual-isotope method (chronic drug treatment) and by the lymph-fistula model (acute injection or infusion of drug). Our lymph data for SaH 58-035 differs only in magnitude compared with that reported by Clark and Tercyak (2), who showed for the first time the inhibitory effect of an ACAT inhibitor using these methods. They reported that the lymph cholesteryl ester output was decreased about 50% (2), whereas, we found a nonsignificant 33% decrease in cholesteryl ester output at the same time point after lipid absorption. In retrospect, it is not surprising that normal cholesterol ab-

sorption occurred in the experiment by Gallo et al. (19) using a dose of only 20 mg/kg for SaH 58-035. The decrease in ACAT activity in mucosal homogenates after the bolus of SaH 58-035 (19) may have been due to inadequate removal of the drug from the lumenal surface or cell membranes not associated with microsomes. Data with CI-976 suggest that even for compounds that inhibit lymphatic cholesteryl ester transport by the bolus method, the infusion of drug is far more effective. We suspect that the significant inhibition of cholesterol absorption by CI-976 at only 12.5 mg/kg using the dual-isotope method is an indication that dosing animals by diet admix is analogous to a "slow infusion," and that different results may have been obtained had we administered the drug by daily gavage in the dual-isotope experiments.

In summary, our data confirm the importance of ACAT for the absorption of dietary cholesterol in the rat. Certainly this alone is of potential relevance as it is thought that cholesterol absorption efficiency regulates cholesterol metabolism and serum cholesterol levels in humans (63). But our data appear to extend the role for ACAT by further emphasizing, as did Erickson et al. (57), that liver ACAT also plays a critical role in the maintenance of cholesterol homeostasis, at least in the rat, and potentially in humans as well. In our view, the following overall events might occur in vivo after CI-976 administration based on in vitro (9) and in vivo (16) data. The compound enters mucosal cells in the proximal gut, selectively inhibits ACAT, and thereby reduces the amount of cholesteryl esters in the core of triglyceride-rich particles. Chronic inhibition of ester production causes accumulation of free cholesterol which decreases the uptake of lumenal free sterol (i.e., disruption of passive uptake process or cholesterol gradient across the membrane). Free cholesterol accumulation in enterocytes also causes inhibition of cholesterol synthesis. Inhibition of intestinal esterification and synthesis of cholesterol results in less cholesterol being transported to the liver. Thus, in cholesterol-fed rats, liver cholesterol content is decreased after chronic treatment and the liver therefore secretes less cholesteryl ester. Active drug reaches the liver via the portal system or in chylomicron remnants to inhibit ACAT, which contributes to the decrease in liver ester storage and secretion and may, in some as yet undefined way, decrease apoB secretion. Unlike the intestine, free cholesterol does not accumulate in this organ but instead is secreted into bile either as cholesterol or bile acids, depending upon the duration of drug treatment. If the primary core lipid of hepatic VLDL is triglyceride rather than cholesteryl ester, ACAT inhibition leads to a decrease in hepatic triglyceride secretion and plasma triglyceride concentration. Drug absorbed via the lymphatics[2] reaches cells of the arterial wall (e.g., macrophages) and fat depots without first passing through the liver. These tissues contain lipoprotein lipase which may facilitate drug uptake from intestinal particles. It is possible, therefore, to demonstrate a direct, antiatherosclerotic effect. Thus, data exists to support the intestine, liver, and arterial wall as drug targets that relate directly or indirectly to the atherogenic process.

Manuscript received 11 May 1992 and in revised form 9 September 1992.

[2] P.J. Martin and D.J. Hauss (Pharmaceutics Department, Parke-Davis) have determined that lymphatically absorbed CI-976 in the rat accounts for at least half of the area under the concentration-time curve (AUC) over a 14-h period after administration of a single dose in aqueous suspension.

REFERENCES

1. Fukushima, H., S. Aono, Y. Nakamura, M. Endo, and T. Imai. 1969. The effect of N-(alpha-methylbenzyl)linoleamide on cholesterol metabolism in rats. *J. Atheroscler. Res.* **10:** 403–414.
2. Clark, S. B., and A. M. Tercyak. 1984. Reduced cholesterol transmucosal transport in rats with inhibited mucosal acyl CoA:cholesterol acyltransferase and normal pancreatic function. *J. Lipid Res.* **25:** 148–159.
3. Harnett, K. M., C. T. Walsh, and L. Zhang. 1989. Effects of Bay o 2752, a hypocholesterolemic agent, on intestinal taurocholate absorption and cholesterol esterification. *J. Pharm. Exp. Ther.* **251:** 502–509.
4. Tso, P., K. M. Morshed, and D. F. Nutting. 1991. Importance of acyl CoA:cholesterol acyltransferase (ACAT) in the esterification of cholesterol by enterocytes. *FASEB J.* **5:** A709.
5. Windler, E., W. Rucker, J. Greeve, H. Reimitz, and H. Greten. 1990. Influence of the acyl-coenzyme A:cholesterol acyltransferase inhibitor octimibate on cholesterol transport in rat mesenteric lymph. *Arzneim. Forsch./Drug Res.* **40:** 1108–1111.
6. Heider, J. G., C. E. Pickens, and L. A. Kelly. 1983. Role of acyl CoA:cholesterol acyltransferase in cholesterol absorption and its inhibition by 57-118 in the rabbit. *J. Lipid Res.* **24:** 1127–1134.
7. Largis, E. E., C. H. Wang, V. G. DeVries, and S. A. Schaffer. 1989. CL 277,082: a novel inhibitor of ACAT-catalyzed cholesterol esterification and cholesterol absorption. *J. Lipid Res.* **30:** 681–690.
8. Schnitzer-Polokoff, R., D. Compton, G. Boykow, H. Davis, and R. Burrier. 1991. Effects of acyl-CoA:cholesterol acyltransferase inhibition on cholesterol absorption and plasma lipoprotein composition in hamsters. *Comp. Biochem. Physiol.* **99A:** 665–670.
9. Field, F. J., E. Albright, and S. Mathur. 1991. Inhibition of acylcoenzyme A:cholesterol acyltransferase activity by PD 128042: effect on cholesterol metabolism and secretion in CaCo-2 cells. *Lipids.* **26:** 1–8.
10. Roth, B. D., C. J. Blankley, M. L. Hoefle, A. Holmes, W. H. Roark, B. K. Trivedi, A. D. Essenburg, K. A. Kieft, B. R. Krause, and R. L. Stanfield. 1992. Inhibitors of acyl-CoA:cholesterol. 1. Identification and structure-activity relationships of a novel series of fatty acid anilide hypocholesterolemic agents. *J. Med. Chem.* **35:** 1609–1617.
11. Field, F. J., and R. G. Salome. 1982. Effect of dietary fat saturation, cholesterol and cholestyramine on acyl-

CoA:cholesterol acyltransferase activity in rabbit intestinal microsomes. *Biochim. Biophys. Acta.* **712:** 557–570.
12. Rogers, J. B. 1969. Assay of acyl-CoA:monoglyceride acyltransferase from rat small intestine using continuous recording spectrophotometry. *J. Lipid Res.* **10:** 427–432.
13. Gallo, L. L. 1981. Sterol ester hydrolase from rat pancreas. *Methods Enzymol.* **71:** 664–674.
14. Stokke, K. T., and K. R. Norum. 1971. Determination of lecithin:cholesterol acyltransfer in human blood plasma. *Scand. J. Clin. Lab. Invest.* **27:** 21–27.
15. Hosie, L., L. D. Sutton, and D. M. Quinn. 1987. *p*-Nitrophenyl and cholesteryl-N-alkyl carbamates as inhibitors of cholesterol esterase. *J. Biol. Chem.* **262:** 260–264.
16. Bocan, T. M. A., S. Bak-Mueller, P. D. Uhlendorf, R. S. Newton, and B. R. Krause. 1991. Comparison of CI-976, an ACAT inhibitor, and selected lipid-lowering agents for antiatherosclerotic activity in iliac-femoral and thoracic aortic lesions. A biochemical, morphological and morphometric evaluation. *Arterioscler. Thromb.* **11:** 1830–1843.
17. Burstein, M., H. R. Scholnick, and R. Morfin. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11:** 583–595.
18. Kieft, K. A., T. M. A. Bocan, and B. R. Krause. 1991. Rapid on-line determination of cholesterol distribution among plasma lipoproteins after high-performance gel filtration chromatography. *J. Lipid Res.* **32:** 859–866.
19. Gallo, L. L., J. A. Wadsworth, and G. V. Vahouny. 1987. Normal cholesterol absorption in rats deficient in intestinal acyl coenzyme A:cholesterol acyltransferase activity. *J. Lipid Res.* **28:** 381–387.
20. Rifai, N., and M. E. King. 1986. Immunoturbidemetric assays for apolipoproteins A, A-I, A-II and B in serum. *Clin. Chem.* **32:** 957–961.
21. Windler, E. E., P. T. Kovanen, Y-S. Chao, M. S. Brown, R. J. Havel, and J. L. Goldstein. 1980. The estradiol-stimulated lipoprotein receptor of rat liver. A binding site that mediates the uptake of rat lipoproteins containing apoproteins B and E. *J. Biol. Chem.* **255:** 10464–10471.
22. Davis, R. A., and P. S. Roheim. 1978. Pharmacologically induced hypolipidemia. The ethinyl estradiol-treated rat. *Atherosclerosis.* **30:** 293–299.
23. Davis, R. A., R. Showalter, and F. Kern. 1978. Reversal by Triton WR-1339 of ethynyloestradiol-induced hepatic cholesterol esterification. *Biochem. J.* **174:** 45–51.
24. Strobl, W., N. L. Gorder, G. A. Fienup, Y. Lin-Lee, A. M. Gotto, and W. Patsch. 1989. Effect of sucrose diet on apolipoprotein biosynthesis in rat liver. Increase in apolipoprotein E gene transcription. *J. Biol. Chem.* **264:** 1190–1194.
25. Turley, S. D. and J. M. Dietschy. 1979. Regulation of biliary cholesterol output in the rat: dissociation from the rate of hepatic cholesterol synthesis, the size of the hepatic cholesteryl ester pool, and the hepatic uptake of chylomicron cholesterol. *J. Lipid Res.* **20:** 923–934.
26. Turley, S. D., and J. M. Dietschy. 1978. Re-evaluation of the 3α-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J. Lipid Res.* **19:** 924–928.
27. Fand, I., and W. P. McNally. 1981. The technique of whole-body autoradiography. *In* Morphological Techniques for Gerontology. J. E. Johnson, Jr., editor. CRC Press, Boca Raton, FL. 1–28.
28. Zilversmit, D. B. 1972. A single blood sample dual isotope method for the measurement of cholesterol absorption in rats. *Proc. Soc. Exp. Biol. Med.* **140:** 862–865.
29. Cayen, M. N., and D. Dvornik. 1979. Effect of diosgenin on lipid metabolism in rats. *J. Lipid Res.* **20:** 162–174.
30. Krause, B. R., C. H. Sloop, C. K. Castle, and P. S. Roheim. 1981. Mesenteric lymph apolipoproteins in control and ethinyl estradiol-treated rats: a model for studying apolipoproteins of intestinal origin. *J. Lipid Res.* **22:** 610–619.
31. Slayback, J. R. B., L. W. Y. Cheung, and R. P. Geyer. 1977. Quantitative extraction of microgram amounts of lipid from cultured human cells. *Anal. Biochem.* **83:** 372–384.
32. Christie, W. W. 1985. Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. *J. Lipid Res.* **26:** 507–512.
33. Natori, K., Y. Okazaki, T. Nakajima, T. Hirohashi, and S. Aono. 1986. Mechanism of the inhibition of cholesterol absorption by DL-melinamide: inhibition of cholesterol esterification. *Jpn. J. Pharm.* **42:** 517–523.
34. DeVries, V. G., S. A. Schaffer, E. E. Largis, M. D. Dutia, C. H. Wang, J. D. Bloom, and A. S. Katocs. 1986. Potential antiatherosclerotic agents. 5. An acyl-CoA:cholesterol O-acyltransferase inhibitor with hypocholesterolemic activity. *J. Med. Chem.* **29:** 1131–1133.
35. Ross, A. C., K. J. Go, J. G. Heider, and G. H. Rothblat. 1984. Selective inhibition of acyl coenzyme A:cholesterol acyltransferase by compound 58-035. *J. Biol. Chem.* **259:** 815–819.
36. Veldhuis, J. D., J. F. Strauss, S. L. Silavin, L. A. Kolp, P. A. Klase, D. Juchter, and J. Garmey. 1985. The role of cholesterol esterification in ovarian steroidogenesis: studies in cultured swine granulosa cells using a novel inhibitor of acyl coenzyme A:cholesterol acyltransferase. *Endocrinology.* **116:** 25–30.
37. Rucker, W., G. Prop, H. Wetzig, E. Huff, R. Niemann, and E. Graf. 1985. Octimibate, a triphenylimidazole-derivative with hypolipidemic and antiatherosclerotic effects in rabbits and mini-pigs. 7th International Symposium on Atherosclerosis, Melbourne (poster presentation).
38. Hamelehle, K. L., B. R. Krause, and R. Homan. 1991. Differentiation between cholesterol and retinol esterification in CaCo-2 cells by CI-976, an inhibitor of acylcoenzyme A:cholesterol acyltransferase. *J. Cell Biol.* **111:** 190a.
39. Ohata, I., N. Sakamoto, K. Nagano, and H. Maeno. 1984. Low density lipoprotein-lowering and high density lipoprotein-elevating effects of nicardipine in rats. *Biochem. Pharmacol.* **33:** 2199–2205.
40. Uchida, K., Y. Nomura, and N. Takeuchi. 1980. Effects of cholic acid, chenodeoxycholic acid, and their related bile acids on cholesterol, phospholipid and bile acid levels in serum, bile and feces of rats. *J. Biochem.* **87:** 187–194.
41. Davis, R. A., and F. Kern. 1976. Effects of ethinyl estradiol and phenobarbital on bile acid synthesis and biliary bile acid and cholesterol excretion. *Gastroenterology.* **70:** 1130–1135.
42. Imai, Y., S. Kawata, M. Inada, S. Mioshi, Y. Minami, Y. Matsuzawa, K. Uchida, and S. Tauri. Effect of cholestyramine on bile acid metabolism in rats. *Lipids.* **22:** 513–516.
43. Fukushima, H., and H. Nakatani. 1969. Cholesterol-lowering effects of dl-N-(alpha-methylbenzyl)-linoleamide and its optically active isomers in cholesterol-fed animals. *J. Atheroscler. Res.* **9:** 65–71.
44. Krause, B. R., and R. J. Newton. 1985. Apolipoprotein changes associated with the plasma lipid-regulating activity of gemfibrozil in cholesterol-fed rats. *J. Lipid Res.* **26:** 940–949.
45. Kam, N. T. P., E. Albright, S. N. Mathur, and F. J. Field. 1989. Inhibition of acylcoenzyme A:cholesterol acyltrans-

ferase activity in CaCo-2 cells results in intracellular triglyceride accumulation. *J. Lipid Res.* **30:** 371–377.
46. Balasubramaniam, S., L. A. Simons, S. Chang, P. D. Roach, and P. J. Nestel. 1990. On the mechanism by which an ACAT inhibitor (CL 277,082) influences plasma lipoproteins in the rat. *Atherosclerosis.* **82:** 1–5.
47. Nervi, F., M. Bronfman, W. Allalon, E. Depiereux, and R. Del Pozo. 1984. Regulation of biliary cholesterol secretion in the rat. Role of hepatic cholesterol esterification. *J. Clin. Invest.* **74:** 2226–2237.
48. Sampson, W. J., R. A. Suffolk, P. Bowers, J. D. Houghton, K. M. Botham, and K. E. Suckling. 1987. The role of acyl-CoA:cholesterol acyltransferase in the metabolism of free cholesterol to cholesteryl esters or bile acids in primary cultures of rat hepatocytes. *Biochim. Biophys. Acta.* **920:** 1–8.
49. Drevon, C. A., S. C. Engelhorn, and D. Steinberg. 1980. Secretion of very low density lipoproteins enriched in cholesteryl esters by cultured rat hepatocytes during stimulation of intracellular cholesterol esterification. *J. Lipid Res.* **21:** 1065–1071.
50. Davis, R. A., M. M. McNeal, and R. L. Moses. 1982. Intrahepatic assembly of very low density lipoprotein. Competition by cholesterol esters for the hydrophobic core. *J. Biol. Chem.* **257:** 2634–2640.
51. Noel, S. P., L. Wong, P. J. Dolphin, L. Dory, and D. Rubenstein. 1979. Secretion of cholesterol-rich lipoproteins by perfused livers of hypercholesterolemic rats. *J. Clin. Invest.* **64:** 674–683.
52. Khan, B., H. G. Wilcox, and M. Heimberg. 1989. Cholesterol is required for secretion of very-low-density lipoprotein by rat liver. *Biochem. J.* **259:** 807–816.
53. Fungwe, T. V., L. Cagen, H. G. Wilcox, and M. Heimberg. 1992. Regulation of hepatic secretion of very low density lipoprotein by dietary cholesterol. *J. Lipid Res.* **33:** 179–191.
54. Cianflone, K. M., Z. Yasruel, M. A. Rodriguez, D. Vas, and A. D. Sniderman. 1990. Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge. *J. Lipid Res.* **31:** 2045–2055.
55. Krause, B. R., K. Kieft, B. Auerbach, R. Stanfield, R. Bousley, and C. Bisgaier. 1991. ACAT inhibition by CI-976 lowers LDL-cholesterol in rabbits fed a cholesterol-free diet. *Circulation.* **84:** II-138.
56. Carr, T. P., and L. L. Rudel. 1990. Partial inhibition of ACAT decreases apoB secretion by the liver of African green monkeys. *Arteriosclerosis.* **10:** 823a.
57. Erickson, S. K., M. A. Shrewsbury, C. Brooks, and D. J. Meyer. 1980. Rat liver acyl-coenzyme A:cholesterol acyltransferase: its regulation in vivo and some of its properties in vitro. *J. Lipid Res.* **21:** 930–941.
58. Del Pozo, R., F. Nervi, C. Corvarrubias, and B. Ronco. 1983. Reversal of progesterone-induced biliary cholesterol output by dietary cholesterol and ethynylestradiol. *Biochim. Biophys. Acta.* **753:** 164–172.
59. Erickson, S. K., and A. D. Cooper. 1980. Acyl-coenzyme A:cholesterol acyltransferase in human liver. In vitro detection and some characteristics of the enzyme. *Metabolism.* **29:** 991–996.
60. Smith, J. L., J. de Jersey, S. P. Pillay, and I. R. Hardie. 1986. Hepatic acyl-CoA:cholesterol acyltransferase. Development of a standard assay and determination in patients with cholesterol gallstones. *Clin. Chim. Acta.* **158:** 271–282.
61. Einarsson, K., L. Benthin, S. Ewerth, G. Hellers, D. Ståhlberg, and B. Angelin. 1989. Studies on acyl-coenzyme A:cholesterol acyltransferase activity in human liver microsomes. *J. Lipid Res.* **30:** 739–746.
62. Bisgaier, C. L., M. V. Siebenkas, M. L. Brown, A. Inazu, J. Koizumi, H. Mabuchi, and A. R. Tall. 1991. Familial cholesteryl ester transfer protein deficiency is associated with triglyceride-rich low density lipoproteins containing cholesteryl esters of probable intracellular origin. *J. Lipid Res.* **32:** 21–33.
63. Miettinen, T. A., and Y. A. Kesäniemi. 1988. Cholesterol absorption regulates cholestrol metabolism and within-population variation of serum cholesterol. *In* Hyperlipidaemia and Atherosclerosis. K. E. Suckling and P. H. E. Groot, editors. Academic Press, London. 73–82.